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Genetic differentiation in four species of *Apodemus* from Southern Europe: *A. sylvaticus, A. flavicollis, A. agrarius* and *A. mystacinus* (Muridae, Rodentia)

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Abstract

Genic variability was estimated for four *Apodemus* species from Southern Europe by electrophoresis at 20 loci. Genic divergence data agree well with the subgeneric classification, *A. agrarius* (subgenus *Apodemus*) being very distant from the three other species (subgenus *Sylvaemus*), as was previously shown by Gemmeke (1980) using 11 loci. However, the large divergence between these two groups suggests that their taxonomic relationships should be revised. The morphological overlap between *A. sylvaticus* and *A. flavicollis* is confirmed throughout Southern Europe which stresses the use of biochemical methods for unambiguous identification of specimens. The karyological analysis of samples of *A. sylvaticus, A. flavicollis* and *A. agrarius* revealed chromosomal variation in only one individual (*A. flavicollis* with 2 n = 49 in Bulgaria).

Introduction

The genus *Apodemus* is one of the most widespread noncommensal rodent groups in the Paleartic. In the Western part of their range, the use of biochemical genetics has proven extremely useful in unambiguously discriminating morphologically similar species. This has been the case for *A. sylvaticus* and *A. flavicollis*, in particular, which although easily distinguishable in Central and Northern Europe, show a morphological overlap in the more southern areas. That this overlap is due to clinal variation in size and pelage color following opposite trends in both species and not to hybridization, has been shown by a number of authors (Niethammer and Krapp 1978; Gemmeke 1980; Benmehdi et al. 1980; Nascetti and Filippucci 1984). This discrete morphological variation seems to be a general trait within the subgenus *Sylvaemus* (*A. mystacinus* excepted), although genic differentiation as measured by electrophoretic methods is quite extensive. Recent studies of more eastern populations suggest that this subgenus will most likely reveal a complex of species (Niethammer 1969; Darviche et al. 1979; Gemmeke and Niethammer 1982).

Karyotypic variability is also well documented within the subgenera *Sylvaemus* and *Apodemus*. All species carry 48 chromosomes but differ in the NF number showing that chromosomal evolution has proceeded mainly by pericentric inversions (Kral 1970; Soldatovic et al. 1975; for a review see Zima and Kral 1984).

The evolutionary relationship between *Apodemus* species was previously investigated at 11 loci by Gemmeke (1980). The genetic differentiation between *Apodemus* species belonging to the two subgenera *Sylvaemus (A. sylvaticus, A. flavicollis, A. mystacinus)* and *Apodemus (A. agrarius)* is here extended to populations from Greece, Bulgaria and Spain for which morphological, chromosomal and allozymic data at 20 loci are presented.

Material and methods

Specimens belonging to four species of the genus *Apodemus* (*A. sylvaticus, A. flavicollis, A. mystacinus* and *A. agrarius*) were live-trapped in localities (see Figure 1 for names) from five southern European countries. Nine populations were analyzed for genic variability: France (1), Italy (2), Greece (3, 4, 5, 6), Spain (7) and Bulgaria (8). Starch gel electrophoresis techniques are described in Pasteur et al. (1987). A total of 20 loci were analysed: alcohol dehydrogenase *(Adh)*, alpha-glycerophosphate dehydrogenase *(alpha-Gpd)*, albumin *(Alb)*, amylase *(Amy-1)*, glutamate oxaloacetate transaminase *(Got-1)*, glucose phosphate isomerase *(Gpi)*, hemoglobin *(Hbb)*, isocitrate dehydrogenase (*Idh-1* and *Idh-2*), lactate dehydrogenase (*Ldh-1* and *Ldh-2*, respectively coding for the A and B subunits; see Engel et al. 1973), the regulator gene for *Ldh-2* in red cells *(Ldr)*, NAD-dependent malate dehydrogenase (*Mdh-1* and *Mdh-2*), NADP-dependent malate dehydrogenase *(Mod-1)*, nucleoside phosphorylase *(Np)*, phosphoglucose dehydrogenase *(Pgd)*, phosphoglucomutase *(Pgm)*, sorbitol dehydrogenase *(Sdh)* and superoxide dismutase *(Sod)*. Allelic designations were determined by comparison to the mobility of the most frequent allele in *A. sylvaticus* which was arbitrarily assigned the value 100. The HBB pattern in *Apodemus* consists in two spots, the slower one showing no variation, so the genetic variability scored refers only to variation in mobility of the faster migrating spot. Genetic variability measures, genetic distances (Nei 1978) and the UPGMA phenogram were computed and performed using the BIOSYS-1 program of Swofford and Selander (1981).

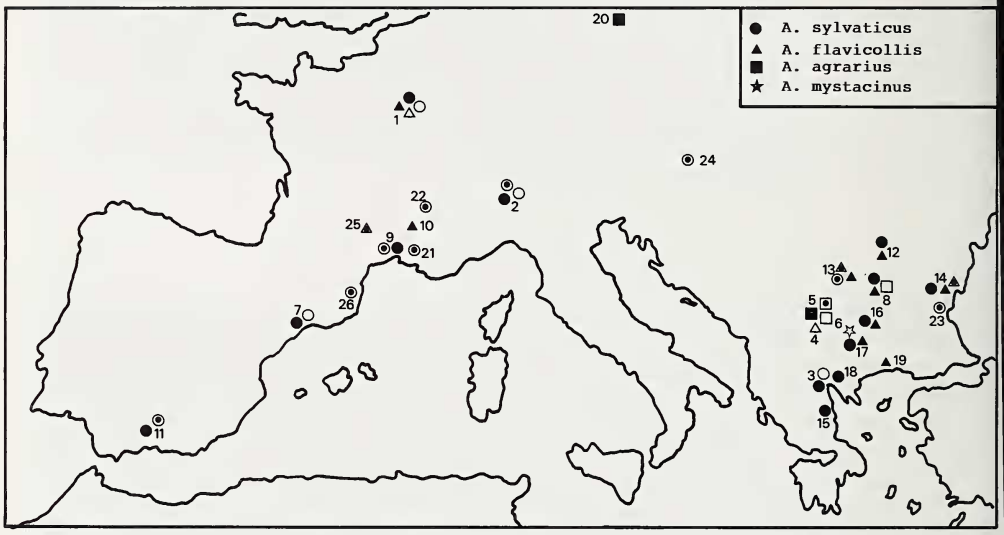

Fig. 1. Distribution of sampled localities and technique of analysis: empty symbols = allozymes; dark symbols = morphology; dotted symbols = chromosomes. Localities: France (1. St Cyr-les-Colons, 9. Gardiole, 10. Avène, 21. Mauguio, 22. Iseron, 25. Cuxac, 26. Banyuls); Italy (2. Ticino region near Pavia); Greece (3. Gallikos, 4. Strimonikon, 5. Doirani, 6. Prosotsani hills, 16. Aggitis river, 17. Prosotsani river, 18. University farm Thessaloniki, 19. Avas); Spain (7. Calonge, 11. Granada); Bulgaria (8. Plovdiv, 12. Karnobat, 13. Vrania, 14. General Toshevo, 23. Orizare); Germany (20. Lübeck) and Austria (24. Burgenland)

Morphological data were collected on specimens from France (1, 9, 10), Italy (2), Spain (7, 11), Bulgaria (8, 12, 13, 14), Greece (3, 15, 16, 17, 18, 19) and Germany (20).

The chromosomal study was performed on field mice from different localities: *A. sylvaticus* from France (9:1 male, 21:1 male, 22:1 female, 26:1 male), Spain (11:1 female), Bulgaria (13:1 female, 23:1 female) and Austria (24:1 female); *A. flavicollis* from France (25:1 female) and Bulgaria (14:1 male, 13:1 male); *A. agrarius* from Greece (4:1 male, 1 female). The karyotypes were established using the classical air-drying technique. No results were available for *A. mystacinus*.

In both the morphological and chromosomal study, all mice, except for *A. agrarius* and *A. mystacinus* which were unambiguously identified in the field, were ascribed to *A. sylvaticus* or *A. flavicollis* on the basis of their *LDH-2* alleles. The specimens are deposited as pickled carcasses at the Institut des Sciences de l'Evolution.

Table 1. **Allele frequencies at the 19 variable loci**

		Species / Locality							
Locus		*AS* FR	*AS* IT	*AS* GR	*AS* SP	*AF* FR	*AA* GR	*AA* BU	*AM* GR
Adh	(N)	30	7	15	9	17	8	9	1
	f	0.62	0.50	0.70	0.72	0.00	1.00	1.00	0.00
	s	0.38	0.50	0.30	0.28	1.00	0.00	0.00	1.00
Gpd	(N)	33	10	24	10	19	8	9	1
	100	0.97	0.80	0.75	0.95	0.97	0.00	0.00	0.00
	70	0.02	0.05	0.25	0.05	0.00	0.00	0.00	0.00
	120	0.01	0.15	0.00	0.00	0.03	1.00	1.00	1.00
Alb	(N)	29	10	24	10	19	8	9	3
	100	1.00	1.00	1.00	1.00	0.00	0.00	0.00	1.00
	95	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00
	98	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00
Amy-1	(N)	28	1	24	9	19	6	1	3
	a	0.98	1.00	0.02	0.22	0.03	0.00	0.50	0.00
	b	0.02	0.00	0.98	0.78	0.97	1.00	0.50	0.00
	c	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00
Got-1	(N)	33	10	24	10	19	8	9	1
	100	0.97	1.00	1.00	1.00	1.00	0.00	0.00	1.00
	95	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	98	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00
Gpi	(N)	30	10	24	9	16	8	9	3
	100	0.98	0.95	1.00	1.00	0.59	0.00	0.00	1.00
	80	0.02	0.00	0.00	0.00	0.41	0.00	0.00	0.00
	200	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00
	30	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00
Hbb	(N)	19	7	24	6	2	8	9	3
	100	1.00	1.00	1.00	1.00	0.00	1.00	1.00	0.00
	120	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00
	140	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00
Idh-1	(N)	33	10	24	9	19	8	9	1
	100	1.00	1.00	0.77	1.00	0.00	0.00	0.00	0.50
	120	0.00	0.00	0.23	0.00	1.00	0.00	0.00	0.00
	130	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00
	125	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.50
Ldh-1	(N)	33	10	24	10	19	8	9	3
	100	1.00	1.00	1.00	1.00	1.00	0.06	0.00	0.00
	90	0.00	0.00	0.00	0.00	0.00	0.94	1.00	0.00
	250	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00
Ldh-2	(N)	33	10	24	10	19	8	9	1
	100	1.00	0.95	1.00	1.00	0.00	0.00	0.00	0.00
	115	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00
	95	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00
	90	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00
Ldr	(N)	22	7	23	9	19	8	9	3
	a	0.37	0.00	0.00	0.47	0.00	1.00	1.00	0.82
	b	0.63	1.00	1.00	0.53	1.00	0.00	0.00	0.18
Mdh-1	(N)	33	10	24	10	19	8	9	1
	100	1.00	1.00	1.00	1.00	1.00	0.00	0.00	1.00
	70	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00
Mdh-2	(N)	33	10	24	10	19	8	9	1
	100	1.00	1.00	1.00	0.65	1.00	1.00	1.00	1.00
	80	0.00	0.00	0.00	0.35	0.00	0.00	0.00	0.00

Table 1 (continued)

Locus		Species / Locality *AS* FR	*AS* IT	*AS* GR	*AS* SP	*AF* FR	*AA* GR	*AA* BU	*AM* GR
Mod-1	(N)	33	10	24	10	19	8	9	1
	100	0.95	0.55	1.00	1.00	0.00	0.00	0.00	0.00
	110	0.05	0.40	0.00	0.00	0.00	0.00	0.00	0.00
	67	0.00	0.00	0.00	0.00	0.92	0.00	0.00	0.00
	30	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00
	90	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00
	50	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00
	120	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00
Np	(N)	31	10	24	10	18	8	9	3
	100	1.00	1.00	1.00	1.00	0.00	0.00	0.00	0.00
	80	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00
	90	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00
	110	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00
Pgd	(N)	31	10	24	10	18	8	9	3
	100	0.98	1.00	1.00	1.00	0.97	1.00	1.00	0.00
	110	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	70	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00
	130	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00
Pgm	(N)	32	10	23	10	14	8	9	1
	100	0.79	1.00	1.00	1.00	1.00	0.50	0.67	0.50
	120	0.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	80	0.08	0.00	0.00	0.00	0.00	0.06	0.00	0.50
	70	0.00	0.00	0.00	0.00	0.00	0.44	0.33	0.00
Sdh	(N)	31	8	14	9	19	8	9	1
	100	0.98	1.00	1.00	1.00	1.00	0.00	0.00	0.00
	110	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	90	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00
	80	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00
	120	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00
Sod	(N)	33	10	24	10	19	8	9	1
	100	1.00	1.00	1.00	1.00	0.00	0.00	0.00	0.00
	70	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00
	110	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00

AS = A. sylvaticus; AF = A. flavicollis; AA = A. agrarius; AM = A. mystacinus. FR = France; IT = Italy; GR = Greece; SP = Spain; BU = Bulgaria.

Results

Genetic variability and differentiation

Genetic variability parameters (Table 2) were computed from the allelic frequencies (Table 1). Only one locus *(Idh-2)* was found monomorphic for the same allele in the four *Apodemus* species. Twelve loci (*Alb, Amy-1, Got-1, c+l.c, Hbb, Idh-1, Ldh-1, Ldh-2, Mdh-1, Pgd, Sdh* and *Sod*) discriminated at least two of the four species, whereas two loci were species diagnostic: *Mod-1* and *Np*.

All variability parameters were highest for *A. sylvaticus* (mean H = 0.08, mean $P_{0.05}$ = 21 % and mean A = 1.3) than for the other three species which ranged between 10 %–15 % for the rate of polymorphism and 4–6 % for mean heterozygosity. However, where sample sizes are small, this measure of variability may not be representative of the species as a whole.

Genetic distances (Table 3) calculated according to NEI (1978) were used to generate a UPGMA phenogram (Fig. 2). The latter agrees well with expectations in that *A. sylvaticus* and *A. flavicollis* cluster together, and both of these with *A. mystacinus*. *A. agrarius*, on the other hand is set well apart from the first three species, yielding a mean genetic distance of 1.28. Intraspecific genetic distances were computed for *A. sylvaticus* and *A. agrarius* only and yielded respectively a mean of 0.045 and 0.067 which falls within the value generally recorded for subspecific genetic differentiation.

Table 2. **Genetic variability measures**

Locality	N	A	P	H
A. sylvaticus				
France (1)	30	1.6	20	0.08
Italy (2)	9	1.3	25	0.08
Greece (3)	23	1.2	15	0.06
Spain (7)	9	1.3	25	0.09
Mean		*1.3*	*21*	*0.08*
A. flavicollis				
France (1)	17	1.3	10	0.04
A. agrarius				
Greece (5)	8	1.1	10	0.03
Bulgaria (8)	9	1.1	10	0.07
Mean		*1.1*	*10*	*0.05*
A. mystacinus				
Greece (6)	2	1.1	15	0.06

A = mean number of alleles; P = % of polymorphic loci (5 % level); H = mean heterozygosity (Nei 1978 except for *A. mystacinus* for which the sample size was too small). Numbers in parenthesis refer to localities.

Morphological discrimination

Morphological data are presented in Table 4 and Figure 3. Whereas discrimination of *A. agrarius* and *A. mystacinus* on morphological grounds is immediate, this is not the case between *A. sylvaticus* and *A. flavicollis* which show a large overlap in body lengths. Additionally, although the presence of a complete collar is species diagnostic, its absence is not since 35 % of the *A. flavicollis* we captured exhibited only a more or less large chest spot in lieu of a collar.

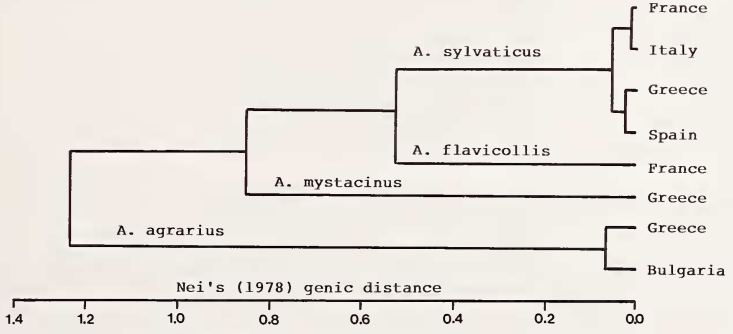

Fig. 2. UPGMA phenogram computed from genic distances

Chromosomal variability

The chromosomal analysis of specimens from each species corresponded to results from previous studies (Kral 1970; Soldatović et al. 1975; Bekasova et al. 1980; Zima 1984). The karyotypes of *A. sylvaticus* and *A. flavicollis* both carried 48 acrocentric chromosomes except for the *A. flavicollis* from Bulgaria (14. General Toshevo) which had 49 chromosomes showing an additional small acrocentric chromosome. Such variation is common in this species and has been attributed to the presence of supernumerary chromosomes (Soldatović et al. 1975; Zima 1984; Zima and Kral 1984). *A. agrarius* (2n

Table 3. **Genetic distance coefficients** (Nei 1978)

Locality	1	2	3	4	5	6	7	8
1. *AS*–FR	– – –							
2. *AS*–IT	0.016	– – –						
3. *AS*–GR	0.065	0.067	– – –					
4. *AS*–SP	0.039	0.063	0.022	– – –				
5. *AF*–FR	0.601	0.548	0.478	0.554	– – –			
6. *AA*–GR	1.236	1.284	1.115	1.107	1.446	– – –		
7. *AA*–BU	1.133	1.166	1.170	1.128	1.522	0.067	– – –	
8. *AM*–GR	0.876	0.866	0.932	0.908	0.823	1.532	1.512	– – –

AS = *A. sylvaticus*; *AF* = *A. flavicollis; AA* = *A. agrarius; AM* = *A. mystacinus.*
FR = France; IT = Italy; GR = Greece; SP = Spain; BU = Bulgaria.

Table 4. **Morphological data for samples of three *Apodemus* species**
Numbers after countries refer to localities sampled

Locality	N	BL (mm)	TL (mm)	HF (mm)	TL/BL	Chest spot No	Sm	L	Col
A. sylvaticus									
Italy (2)	10	93(3)	77(5)	22(1)	0.84(0.07)	20%	80%	–	–
Bulgaria (8. 12–14)	14	96(6)	81(4)	23(0)	0.86(0.05)	36%	57%	7%	–
Spain (7. 11)	9	93(5)	88(7)	22(1)	0.96(0.07)	44%	56%	–	–
Greece (3. 15–18)	48	107(2)	91(2)	23(0)	0.85(0.02)	81%	16%	3%	–
France (1)	33	84(2)	79(2)	21(1)	0.93(0.02)	33%	39%	27%	
(9)	20	97(2)	84(3)	21(1)	0.87(0.03)	30%	70%	–	–
Mean		*97(2)*	*84(1)*	*22(0)*	*0.88(0.01)*	*53%*	*39%*	*8%*	–
A. flavicollis									
Greece (16–17)	2	97(–)	88(–)	23(–)	0.92(–)	–	100%	–	–
Bulgaria (8. 12–14)	6	98(7)	86(1)	22(2)	0.88(0.08)	–	75%	–	25%
France (10)	20	94(5)	95(4)	23(1)	1.03(0.04)	–	15%	35%	50%
(1)	19	94(5)	99(5)	24(0)	1.06(0.03)	–	–	5%	95%
Mean		*95(3)*	*95(3)*	*23(0)*	*1.01(0.03)*	–	*19%*	*16%*	*65%*
A. agrarius									
Greece (5)	8	112(5)	78(2)	22(1)	0.69(0.02)				
Germany (20)	6	102(5)	70(12)	19(1)	0.69(0.17)				
Mean		*108(4)*	*75(5)*	*21(1)*	*0.69(0.05)*				

BL = body length; TL = tail length; HF = hind foot length; chest spot color: no, small (Sm) or large (L) spot; Col = complete collar. STandard errors in parenthesis.

= 48) showed four pairs of small metacentric chromosomes which increased the NF to 56. No other chromosomal variability was found within samples.

Discussion

Interspecific genetic differentiation at 20 loci is presented for four species of the genus *Apodemus*. Previous studies on these same species were made by Gemmeke (1980) but the electrophoretic survey included only 11 loci. More recent and extensive work was performed by Nascetti and Filippucci (1984) on 27 loci but was restricted to the subgenus *Sylvaemus*. The overview of these three independent studies shows that the

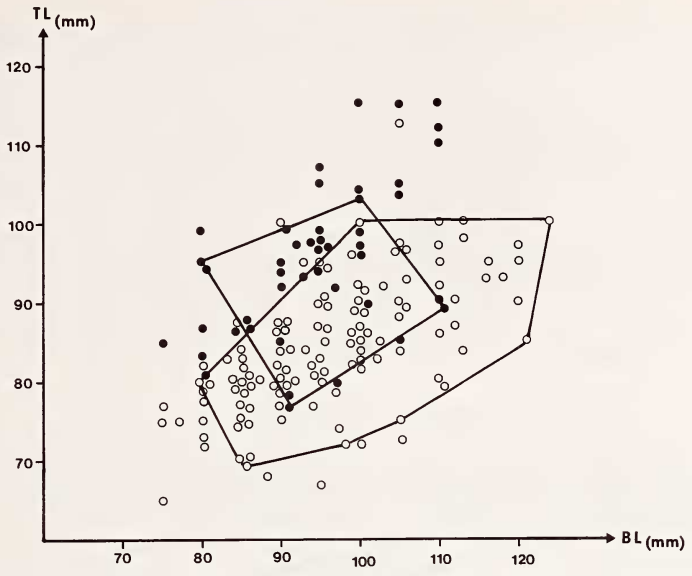

Fig. 3. Body (BL) and tail length (TL) distribution. Empty circle = *A. sylvaticus;* dark circle = *A. flavicollis*. Envelopes show the morphological variability of samples from Bulgaria and Greece for both species

overall specific discrimination (the number of diagnostic loci between two species) is similar and often more important with the set of loci and the electrophoretic buffers we used. This reasoning is based on the assumption that the differences in scoring results are probably more related to the techniques used than to variability of the biological material. These differences in discriminating capacity do not, however, alter the phylogenetic relationships as determined by the genetic distances.

Our results agree with previous work on this genus (Gemmeke 1980) in that *A. sylvaticus*, *A. flavicollis* and *A. mystacinus* belonging to the same subgenus *Sylvaemus* cluster together whereas *A. agrarius*, which represents a different subgenus *(Apodemus)* is exterior to this group.

That morphological discrimination is difficult and even impossible between specimens of *A. sylvaticus* and *A. flavicollis* inhabiting Southern Europe has previously been shown for France (Benmehdi et al. 1980), Italy (Nascetti and Filippucci 1984) and Germany (Engel et al. 1973; Gemmeke 1980) and is here extended to Bulgaria and Greece. Previous results are here again confirmed in that in all cases of morphological ambiguity, the biochemical analysis allowed to assign the specimens to either species and showed the absence of any introgression between them. It is therefore suggested that field specimens be identified by electrophoretic methods (on albumin for example, Debrot and Mermod 1977; Gemmeke 1981) or by using the morphological criteria put forth by Filippucci et al. (1984) which enabled to discriminate at least 95 % of Italian specimens and should be tested elsewhere.

This study shows that the separation of the four *Apodemus* species studied into two subgenera is supported by the biochemical distance data. Within the subgenus *Sylvaemus*, *A. sylvaticus* and *A. flavicollis* are remarkable in that they represent morphologically and chromosomally very similar species with relatively large genic distances.

The particular position of *A. agrarius* is worth commenting on, however. The very important genic distance between this species and those of the subgenus *Sylvaemus* is

probably an underestimate, being at the limit of the discriminating power of electrophoretic methods. In fact, Iskandar and Bonhomme (1984) showed that sequential electrophoresis allowed to uncover 27 % more alleles between the two subgenera whereas no additional variation was revealed between the three species of the subgenus *Sylvaemus*. These data then suggest that *A. agrarius* is probably even more distantly related to the *Sylvaemus* species group than what we indicate herein. Based on these data, we agree with Bonhomme et al. (1985) in suggesting that a taxonomic revision of this group be made. It is probable that the two current subgenera will be elevated to a genus rank since *Apodemus* appeared not to be more closely related to *Sylvaemus* than to other murid (Bonhomme et al. 1985) or even arvicolid (data not based on sequential electrophoresis: Gill et al. 1987) genera. For this analysis, it would be imperative that biochemical data be collected for species belonging to the third subgenus *(Alsomys)* in order to correctly establish the evolutionary relationships within the *Apodemus* complex.

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Zusammenfassung

Genetische Differenzierung bei vier Apodemus-Arten in Südeuropa: A. sylvaticus, A. flavicollis, A. agrarius und A. mystacinus (Muridae, Rodentia)

Die genetische Variabilität von vier südeuropäischen *Apodemus*-Arten wurde durch Elektrophorese von Proteinen geschätzt, die über 20 Genloci kodiert werden. Die nach Nei (1978) berechneten Abstandswerte entsprechen ungefähr den bisherigen Vorstellungen von der abgestuften Verwandtschaft dieser Arten. So unterscheidet sich *A. agrarius* (Untergattung *Apodemus*) beträchtlich von den drei anderen, in der Untergattung *Sylvaemus* zusammengefaßten Arten. *Apodemus sylvaticus* und *A. flavicollis* überschneiden sich in ihren morphologischen Merkmalen in Südeuropa stark, lassen sich aber gelelektrophoretisch stets einwandfrei bestimmen. Alle vier Arten haben gewöhnlich 48 Chromosomen. Nur 1 *A. flavicollis* von 27 aus Bulgarien hatte mit einem kleinen zusätzlichen akrozentrischen Element insgesamt 49 Chromosomen.

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